EXHIBIT 6c

Dosing humans therapeutically, based on packaging information, is 1 gram in a reference human adult (60Kg), an exposure of 16.7mg/Kg. Calculated therapeutic doses based on HEDs are 16.7mg/Kg X 12.3 = 205 mg/Kg (mouse) and 16.7mg/Kg X 6.2 = 104 mg/Kg (rat). Actual therapeutic doses have been determined experimentally in animals for various antinociceptive, pain relief, effects. For example, a therapeutic mouse median effective dose (ED_{50}) is 185.52 mg/Kg (electrical tail stimulation in mice), and a therapeutic rat ED_{50} is 154.8 mg/Kg (Randall-Selitto's test in rats).[83] In this example, the electrical tail stimulation test in mice is within 10% of the calculated mouse HED and the Randall-Selitto's test (paw pressure test) is within 50% of the rat HED. Similarly, the mouse ED_{50} of 186mg/Kg divided by 12.3 = 15.1mg/Kg (HED) and the rat ED_{50} of 155mg/Kg divided by 6.2 = 25mg/Kg (HED), and the package information is 16.7mg/Kg (actual human dose). Remarkably, a human dose is between the experimentally determined median effective doses from these animal models converted to HEDs 15.1-25mg/kg. **Based on these data and calculations, a mouse dose of ~150-200mg/Kg or a rat dose of ~100-150 mg/Kg is therapeutic, as reported from experimental studies and calculated using HED conversions.**

In summary, one should not compare mg/Kg dosing directly between different species. The HED conversion should be used to account for biological and metabolic differences between different species when comparing human and animal studies. These conversions are consistent with industry guidelines and allow reasonable estimates to be calculated from human dosages, for equivalent dosing in research animals, and they can also be used in the reverse, to convert dosages in animal research to human equivalent doses (HED).

THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF ACETAMINOPHEN

Adverse Outcome Pathway for Oxidative Stress and Developmental Impairment in Learning in Memory

The Organization for Economic Co-operation and Development (OECD) is an international organization, in which the U.S. participates through the Environmental Protection Agency, that analyzes causal relationships between chemical exposures and adverse outcomes in humans. The OECD prepared an Adverse Outcome Pathway analysis on a set of chemicals that bind to certain proteins, in particular to glutathione.[88] The results were published December 15, 2022, as AOP 20. This analysis is relevant here because one of the identified stressor chemicals for AOP 20 is acetaminophen, and the analysis concluded that exposure to these chemicals during "brain development" may create "oxidative stress" sufficient to "cause cellular injury and death" that disrupts "the establishment of neuronal connections and networks" and can "lead to functional impairment in learning and memory." AOP 20 Abstract.

This AOP report is titled, "Binding of electrophilic chemicals to SH (thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory."[89] It is AOP 20 in the OECD Series on Adverse Outcome Pathways (Figure 11). This AOP is endorsed by regulatory authorities, including the National Coordinators of the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA). The United States and numerous other countries are member countries for the OECD.[90]

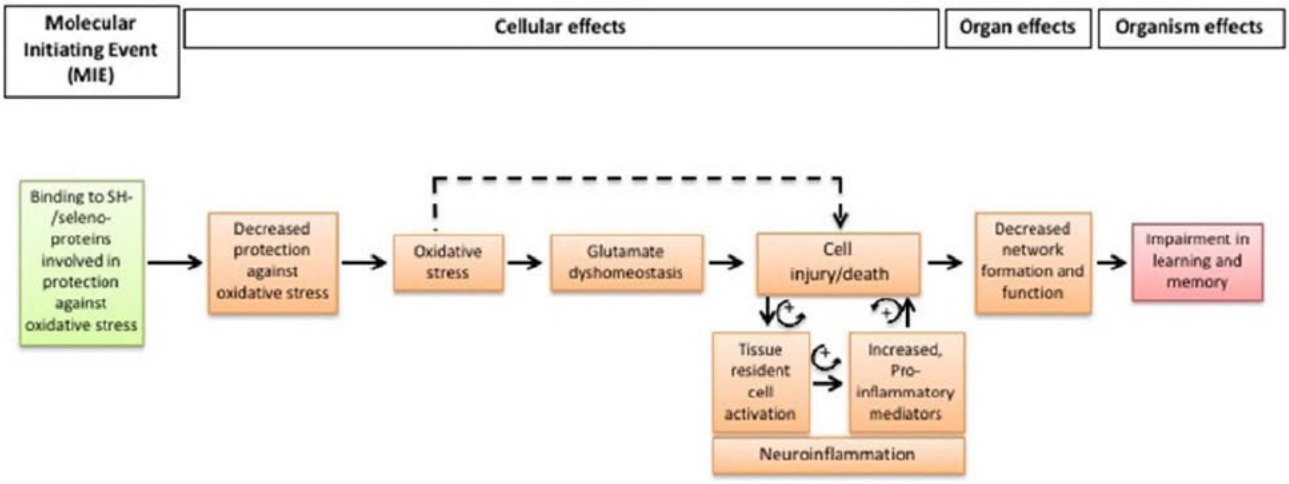

Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory

The abstract for the OECD AOP 20 reads as follows,

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[88] Tschudi-Monnet et al. Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory, OECD Series on Adverse Outcome Pathways, No. 20, OECD Publishing, Paris, 2022. https://doi.org/10.1787/4df0e9e4-en.

[89] Id.

[90] *National co-ordinators of the Test Guidelines programme*, ORG. FOR ECON. CO-OPERATION AND DEV., https://www.oecd.org/chemicalsafety/testing/national-coordinators-test-guidelines-programme.htm.

> This Adverse Outcome Pathway (AOP) describes the linkage between binding to sulfhydryl (SH)-/seleno-proteins involved in protection against oxidative stress and impairment in learning and memory, the Adverse Outcome (AO). Binding to SH-/ seleno-proteins involved in protection against oxidative stress has been defined as the Molecular Initiating Event (MIE). Production, binding and degradation of Reactive Oxygen Radicals (ROS) are tightly regulated, and an imbalance between production and protection may cause oxidative stress, which is common to many toxicity pathways. Oxidative stress may lead to an imbalance in glutamate neurotransmission, which is involved in learning and memory. **Oxidative stress may also cause cellular injury and death. During brain development and in particular during the establishment of neuronal connections and networks, such perturbations may lead to functional impairment in learning and memory.** Neuroinflammation (Resident cell activation; Increased pro-inflammatory mediators) is triggered early in cell injury cascades and is considered as an exacerbating factor. The weight-of-evidence supporting the relationship between the described key events is based mainly on developmental effects observed after an exposure to the heavy metal, mercury, known for its strong affinity to many SH-/seleno-containing proteins, but in particular to those having anti-oxidant properties, such as glutathione (GSH). The overall assessment of this AOP is considered as strong, based on the biological plausibility, the empirical support and on the essentiality of the Key Events (KEs), which are moderate to strong, since blocking, preventing or attenuating an upstream KE is mitigating the downstream KE. The gap of knowledge is mainly due to limited quantitative evaluations, impeding thus the development of predictive models.

AOP 20 identifies acetaminophen as a stressor chemical for which the adverse outcome pathway applies (p 42, Event 1392: Oxidative Stress). The report also indicates that APAP produces oxidative stress as a key event. This is not unexpected, as APAP is known to produce hepatotoxicity via NAPQI and oxidative damage, which can be mitigated by NAC, as indicated above in **Acetaminophen Toxicity**. The OECD AOP 20 report is "based mainly on developmental effects observed after an exposure to the heavy metal, mercury…". The listed prototypical stressors for this AOP are methylmercuric (II) chloride, mercuric chloride, and acrylamide. The AOP describes oxidative stress: (p 43).

> Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell...

Regarding the potential impacts of this oxidative stress, the AOP continues,

> …The brain possesses several key physiological features, such as high O_2 utilization, high polyunsaturated fatty acids content, presence of autooxidable neurotransmitters, and low antioxidant defenses as compared to other organs, that make it highly susceptible to oxidative stress…

The OECD AOP 20 provides a weight-of-evidence analysis for the stressor mercury. By incorporating APAP as a stressor, the AOP provides independent support for a causal relationship between APAP and the identified key events and adverse outcomes. Oxidative stress, cell injury, and decreased neuronal network function are included as key events, and the common adverse outcomes are impairment in learning and memory. These adverse effects are related to, but not specific for, neurodevelopmental

disorder. Specifically, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) defines neurodevelopmental disorders as Intellectual Developmental Disorder (IDD), communication disorders, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), specific learning disorder, and motor disorders.[91]

Regarding the definition and regulatory significance of impairment in learning and memory as an AO (p 97), the AOP also provides guidance,

> A prime example of impairments in learning and memory as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). Most methods are well established in the published literature and many have been engaged to evaluate the effects of developmental thyroid disruption. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD TG 426) as well as OECD TG 443 (OECD, 2018) require testing of learning and memory (USEPA, 1998; OECD, 2007) advising to use the following tests passive avoidance, delayed-matching-to-position for the adult rat and for the infant rat, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, and acquisition and retention of schedule-controlled behaviour. These DNT Guidelines have been deemed valid to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009). Also in the frame of the OECD GD 43 (2008) on reproductive toxicity, learning and memory testing may have potential to be applied in the context of developmental neurotoxicity studies.

This AOP clearly indicates oxidative stress as a KE, that oxidative stress can produce cell injury, cell injury can result in decreased neuronal network function, and thereby cause impairment in learning and memory. The AOP also defines examples of impairments in learning and memory in children and animal models. Regarding the ability of oxidative stress to cause neurodevelopmental disorders, the AOP indicates (p 185) oxidative stress,

> …has also been linked to neurodevelopmental diseases and deficits like autism spectrum disorder and postnatal motor coordination deficits...

Based on this information, there is compelling evidence and support for APAP to cause impairment in learning and memory, but additionally, APAP specific analysis is warranted. This report will therefore apply this AOP framework to APAP, **focusing on the developmental effects observed after an exposure to APAP,** and determine the weight-of-evidence for a causal relationship between APAP and the neurodevelopmental disorders ASD and ADHD.

DNA Oxidation in Reproductive, Developmental, and Neurodevelopmental Toxicity.

APAP and metabolite NAPQI are sufficient to cause oxidative stress and damage in a dose-dependent manner. This oxidative damage affects the normal functioning of cells, leading to changes in cell behavior. When the level of oxidative damage becomes too high, it can result in cell death/apoptosis. The brain is particularly vulnerable to oxidative stress, and during pregnancy, the human brain produces NAPQI due

[91] American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Washington, DC: Author.

to the presence of CYP2E1.[92, 93] Oxidative damage can also impact the development of the nervous system, leading to potential negative effects on learning, memory, and behavior, including neurodevelopmental disorders like ASD and ADHD. Additionally, oxidative damage can affect other organs, including reproductive tissues in males, specifically epididymal cells.[94] Since the central nervous system and reproductive organs develop throughout pregnancy, exposures to APAP that result in oxidative DNA damage can potentially cause reproductive, developmental, and neurodevelopmental toxicity in cells and tissues where APAP and NAPQI have interactions. These outcomes overlap in preclinical testing, as there are interconnected relationships between oxidative DNA damage, reproductive impacts, development, neurodevelopment, reproductive behaviors, and the potential for multi-generational effects and effects on offspring exposed *in utero*.

AOP: Acetaminophen Causes Oxidative Stress and Neurodevelopmental Disorders

This Adverse Outcome Pathway (AOP) describes the linkage between acetaminophen, metabolites of acetaminophen, glutathione, and neurodevelopmental disorders. Acetaminophen is known to undergo metabolism into two metabolites that result in downstream interactions: NAPQI and AM404. NAPQI, a benzoquinone radical, is a toxic metabolite of APAP that reacts with thiols and sulfhydryl (-SH) groups. AM404 is a metabolite of APAP that alters cannabinoid and serotonergic signaling. Both cannabinoid and serotonergic signaling are essential for normal autonomic and neuronal development. In the following sections, I examine the impact of APAP on serotonergic signaling, AM404 on cannabinoid signaling, and NAPQI on oxidative stress for causal physical and chemical interactions that produce impairment in learning, social behavior, focusing, repetitive behavior, and hyperactivity and neurodevelopmental disorders, in particular ASD and ADHD, the Adverse Outcomes (AOs). The resulting AOP outlines a causal pathway initiated by (1) molecular interactions, on to (2) cellular events, to (3) tissue and organ events, to (4) effects on the organism phenotype and behavior.

1. Molecular Interactions

A. Metabolism of Acetaminophen

APAP is metabolized by three major routes and one more recently discovered minor pathway (see **Acetaminophen Metabolism**, above). These metabolic pathways and associated enzymes (proteins) are glucuronidation (UGT1A1, UGT1A6)[95], sulfation (SULT1A1, SULT1A3, SULT1E1)[96], oxidation (CYP2E1)[97], and deacetylation-hydrolase-arachidonic acid conjugation, by arylacetamide deacetylase

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[92] Brzezinski et al. Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. J Pharmacol Exp Ther. 1999 Jun;289(3):1648-53. PMID: 10336564.

[93] Boutelet-Bochan et al. Expression of CYP2E1 during embryogenesis and fetogenesis in human cephalic tissues: implications for the fetal alcohol syndrome. Biochem Biophys Res Commun. 1997 Sep 18;238(2):443-7. doi: 10.1006/bbrc.1997.7296. PMID: 9299528.

[94] Katen et al. Epididymal CYP2E1 plays a critical role in acrylamide-induced DNA damage in spermatozoa and paternally mediated embryonic resorptions. Biol Reprod. 2017 Apr 1;96(4):921-935. doi: 10.1093/biolre/iox021. PMID: 28379345.

[95] Reviewed in Bock and Köhle. UDP-glucuronosyltransferase 1A6: structural, functional, and regulatory aspects. Methods Enzymol. 2005;400:57-75. doi: 10.1016/S0076-6879(05)00004-2. PMID: 16399343.

[96] Adjei et al. Interindividual variability in acetaminophen sulfation by human fetal liver: implications for pharmacogenetic investigations of drug-induced birth defects. Birth Defects Res A Clin Mol Teratol. 2008 Mar;82(3):155-65. doi: 10.1002/bdra.20535. PMID: 18232020.

[97] Reviewed in Tanaka E. In vivo age-related changes in hepatic drug-oxidizing capacity in humans. J Clin Pharm Ther. 1998 Aug;23(4):247-55. doi: 10.1046/j.1365-2710.1998.00164.x. PMID: 9867310.

(AADAC) and fatty acid amide hydrolase (FAAH).[98] The glucuronidation, sulfation, and oxidation pathways have been known since the 1970s,[99] with the role of AADAC on APAP deacetylation-hydrolysis being characterized most recently in 2010,[100] following the identification of arachidonic acid conjugation of PAP by FAAH in 2005.[101]

The relative composition of APAP metabolites results in production of 50-70% APAP-glucuronide, 25-35% APAP-sulfate, 5-15% NAPQI,[102] and 1-2% PAP,[103] 0.001-0.009% AM404,[104] see **Acetaminophen Metabolism**, above. While glucuronide and sulfate maintain the bulk of APAP metabolism and vary within populations, the reported toxic and therapeutic mechanisms of action for APAP are based on the interactions of NAPQI and AM404.

The production of NAPQI from APAP has been a known metabolite and risk factor for oxidative damage and liver necrosis since the 1970-80s.[105] The oxidation of APAP by the cytochrome P450 enzyme (CYP2E1) found in the liver results in the production of NAPQI. Initial reports indicated that CYP2E1, 1A2, and 3A4 were all implicated in the formation of NAPQI.[106] Additional pharmacokinetic and pharmacogenetic studies indicated that CYP2E1 is the primary enzyme involved in APAP oxidation and 1A2 and 3A4 have negligible impacts *in vivo* on APAP metabolism into NAPQI.[107]

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[98] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[99] Levy and Regårdh. Drug biotransformation interactions in man. V. Acetaminophen and salicylic acid. J Pharm Sci. 1971 Apr;60(4):608-11. doi: 10.1002/jps.2600600423. PMID: 4942784.; Mitchell et al. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther. 1973 Oct;187(1):185-94. PMID: 4746326.

[100] Watanabe et al. Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. Drug Metab Dispos. 2010 Sep;38(9):1532-7. doi: 10.1124/dmd.110.033720. Epub 2010 Jun 11. PMID: 20542992.

[101] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[102] Reviewed in Ghanem et al. Acetaminophen from liver to brain: New insights into drug pharmacological action and toxicity. Pharmacol Res. 2016 Jul;109:119-31. doi: 10.1016/j.phrs.2016.02.020. Epub 2016 Feb 26. PMID: 26921661; PMCID: PMC4912877.

[103] Nicholls et al. NMR and HPLC-NMR spectroscopic studies of futile deacetylation in paracetamol metabolites in rat and man. J Pharm Biomed Anal. 1997 Apr;15(7):901-10. doi: 10.1016/s0731-7085(96)01950-4. PMID: 9160256.; Sharma et al. First evidence of the conversion of paracetamol to AM404 in human cerebrospinal fluid. J Pain Res. 2017 Nov 28;10:2703-2709. doi: 10.2147/JPR.S143500. PMID: 29238213; PMCID: PMC5716395.

[104] Muramatsu et al. Metabolism of AM404 From Acetaminophen at Human Therapeutic Dosages in the Rat Brain. Anesth Pain Med. 2016 Jan 17;6(1):e32873. doi: 10.5812/aapm.32873. PMID: 27110534; PMCID: PMC4834746.

[105] Reviewed in Hinson. Reactive metabolites of phenacetin and acetaminophen: a review. Environ Health Perspect. 1983 Mar;49:71-9. doi: 10.1289/ehp.834971. PMID: 6339229; PMCID: PMC1569121.

[106] Lee et al. Role of CYP2E1 in the hepatotoxicity of acetaminophen. J Biol Chem. 1996 May 17;271(20):12063-7. doi: 10.1074/jbc.271.20.12063. PMID: 8662637.; Patten et al. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem Res Toxicol. 1993 Jul-Aug;6(4):511-8. doi: 10.1021/tx00034a019. PMID: 8374050.

[107] Manyike et al. Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin Pharmacol Ther. 2000 Mar;67(3):275-82. doi: 10.1067/mcp.2000.104736. PMID: 10741631.; Zaher et al. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 1998 Sep;152(1):193-9. doi: 10.1006/taap.1998.8501. PMID: 9772215.

B. Downstream Interactions of NAPQI

NAPQI, the primary toxic metabolite of APAP, is a "radical." A radical (also known as reactive oxygen species or ROS) is a molecule with at least one unpaired electron that is highly reactive and can bind with other molecules. When a radical binds with a molecule, the molecule is said to be "oxidized." A common example of oxidation is rust that appears on metals. Antioxidants are molecules that can bind with free radicals and "scavenge" the radicals before they cause damage by oxidation. Oxidative stress occurs in cells when insufficient antioxidant molecules are available to counteract the effect of unstable free radical molecules.

Glutathione is an antioxidant created in the body that serves as a primary cellular defense against free radicals. Glutathione can mitigate the damage caused by NAPQI. A molecule of glutathione that has not been oxidized by a radical is called "reduced" glutathione. Reduced glutathione (GSH) is made from a tripeptide of glutamine, cysteine, and glycine. The GSH molecule can be oxidized at the -SH group on the cysteinyl amino acid, resulting in oxidized glutathione (GSSG), which accounts for its antioxidant properties and interaction with NAPQI. GSH is particularly important for the brain, because brain tissue is highly susceptible to oxidative stress due to its high content of unsaturated fatty acids, high oxygen consumption, and poorly developed oxidative defense mechanisms.

A single 1,000 milligram dose of APAP can produce 50-150 mg (5-15%) of toxic NAPQI radicals. Free radical and reactive oxygen species (ROS) produce an oxidative environment inside a cell and can thereby deplete GSH. When GSH is depleted, free radicals—including NAPQI and other endogenous radicals that normally rely on GSH buffering—are unimpeded and can cause damage that disrupts normal metabolic, cellular, and neurodevelopment of brain tissues in multiple different ways. (In later sections, I describe how NAPQI binds to GSH, proteins, and enzymes and disrupts normal neural development.)

Because ROS are unstable, their presence in cells is difficult to measure directly. In the published AOP (WPHA/WNT Endorsed), the presence of oxidative stress was examined through indirect measurements of ROS damage. These include measuring glutathione (GSH), the ratio of reduced to oxidized GSH (GSH:GSSG), lipid peroxidation using thiobarbituric acid reactive substances (TBARS), and oxidative DNA damage (8-oxo-dG adducts). I describe below the impact of APAP in causing oxidative stress based on these same measurements.

i. APAP and GSH

It has been known since the 1970s that APAP results in depletion of GSH with increasing dosages. GSH reacts with APAP, but at increasing dosages APAP reacts with other tissues and proteins, such as liver proteins in mice (Table 3). Similar results have also been reported in the yolk sac of cultured rat embryos exposed to APAP.[108]

[108] Weeks et al. Acetaminophen toxicity to cultured rat embryos. Teratog Carcinog Mutagen. 1990;10(5):361-71. doi: 10.1002/tcm.1770100502. PMID: 1981948.

Dose of Acetaminophen	Hepatic Glutathione[a, b]	Covalent Binding to Liver Protein[a]
mg/kg	*% of initial level*	*nmol bound/mg protein*
10	100 ± 3	0.05 ± 0.04
25	95 ± 4	0.03 ± 0.04
100	76 ± 8	0.04 ± 0.04
200	41 ± 6	0.08 ± 0.05
375	19 ± 2	0.71 ± 0.13
750	17 ± 2	1.89 ± 0.21

Table 3. Inverse Relationship of Acetaminophen (APAP) Dose and Liver Glutathione (GSH). Injection (IP) of APAP from 10-750mg/Kg results in hepatic GSH depletion.[109]

The interactions of APAP are concentration dependent, as demonstrated by the capacity of APAP to be metabolized and the depletion of GSH by APAP as a function of dose (Figure 12). Unmetabolized APAP was observed in the hepatocyte suspensions after 3 h incubation with > 2mM APAP. This was paralleled by hepatocellular GSH decreasing above 0.5 mM APAP and approaching depletion at ≥ 2 mM APAP. As indicated above, TBARS were also formed at this transition (Figure 12, Right).

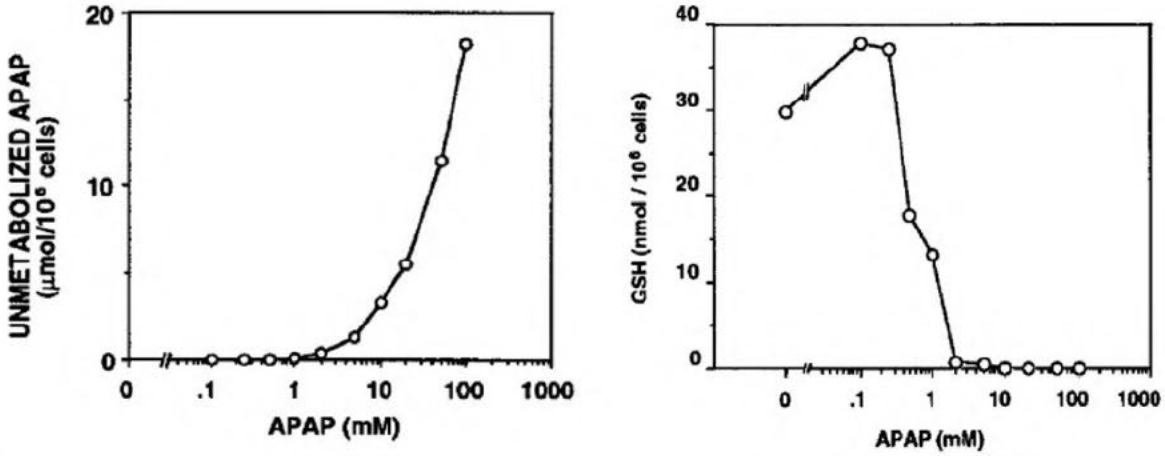

Figure 12. Acetaminophen (APAP) Metabolism and Glutathione (GSH) Interactions. (Left) Unmetabolized APAP in hepatocytes. The unmetabolized APAP concentration in the hepatocyte suspensions incubated for 3 h with APAP was plotted against the initial concentration of APAP. (Right) GSH depletion by APAP in hepatocytes. The hepatocellular GSH concentration was determined in the hepatocytes incubated at 37 °C for 3 h with or without APAP.[111]

ii. APAP and TBARS

Lipid peroxidation is the process that occurs when free radicals attack lipids. A common test for assessing lipid peroxidation is to measure the level of thiobarbituric acid reactive substances (TBARS) formed as a byproduct of lipid peroxidation. APAP at a concentration of 1mM in hepatocytes (liver cells) has been shown to produce a peak concentration of TBARS (Figure 13).[110]

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[109] Mitchell et al. Drug metabolism as a cause of drug toxicity. Drug Metab Dispos. 1973 Jan-Feb;1(1):418-23. PMID: 4149413.
[110] By contrast, APAP has been shown to produce a 50% reduction in TBARS at concentration of 1mM in a sarcoplasmic reticulum model. These data indicate that APAP slows down oxidation in the absence of metabolism. See Dinis et al. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as

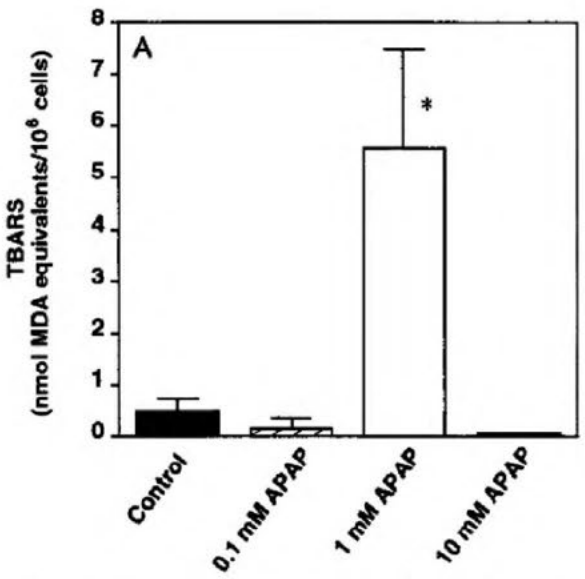

Figure 13. Production of TBARS of Acetaminophen (APAP) is Concentration Dependent. At the low concentration of APAP, 0.1mM, TBARS were not increased. TBARS were produced at 1mM. At the highest concentration, 10mM, unmetabolized APAP was reported to inhibit the formation of TBARS.[111]

In another study, rats exposed to APAP during neurodevelopment at doses equivalent to human therapeutic doses showed significant increased oxidative markers (TBARS) and inflammatory makers.[112] Exposure to APAP (50 mg/kg sc.) on post-natal day 5 (which corresponds to third trimester human development) resulted in the highest increase in TBARS in examined brain tissue.

iii. APAP and DNA Oxidation

Oxidation of DNA can result in oxidized DNA base pairs including 8-hydroxy-deoxyguanosine (8-OH-dG). If not repaired, these oxidized DNA base pairs can cause mutations and/or altered gene expression or epigenetic changes leading to reproductive and developmental toxicity, neurodevelopmental deficits, and cancer.[113] The concentration of oxidized DNA is another measure of oxidative stress. Treatments with APAP at both subtoxic (150mg/kg) and toxic doses (1500mg/Kg and 2000 mg/Kg) were reported to cause a significant increase in DNA oxidation (8-OH-dG) as well as a significant decrease in GSH content in rat liver.**[114]** Increased concentrations of APAP in human cord blood are associated with increased cord

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peroxyl radical scavengers. Arch Biochem Biophys. 1994 Nov 15;315(1):161-9. doi: 10.1006/abbi.1994.1485. PMID: 7979394.

[111] Minamide et al. Spontaneous chemiluminescence production, lipid peroxidation, and covalent binding in rat hepatocytes exposed to acetaminophen. J Pharm Sci. 1998 May;87(5):640-6. doi: 10.1021/js9701014. PMID: 9572917.

[112] Saeedan et al. Effect of early natal supplementation of paracetamol on attenuation of exotoxin/endotoxin induced pyrexia and precipitation of autistic like features in albino rats. Inflammopharmacology. 2018 Aug;26(4):951-961. doi: 10.1007/s10787-017-0440-2. Epub 2018 Jan 11. PMID: 29327281.

[113] Wells et al. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. Toxicol Sci. 2009 Mar;108(1):4-18. doi: 10.1093/toxsci/kfn263. Epub 2009 Jan 6. PMID: 19126598.

[114] Powell et al. Phenotypic anchoring of acetaminophen-induced oxidative stress with gene expression profiles in rat liver. Toxicol Sci. 2006 Sep;93(1):213-22. doi: 10.1093/toxsci/kfl030. Epub 2006 Jun 2. PMID: 16751229; PMCID: PMC1805881.

DNA oxidation (8-OH-dG) level.[115] Independent studies have also reported that urinary 8-OH-dG (DNA oxidation) and 8-OH-G (8-hydroxy-guanosine, RNA oxidation) concentrations are positively correlated with APAP exposures.[116] Another study also reported that urinary concentrations of APAP in pregnant women are associated with higher levels of the selected oxidative stress biomarkers: 8-OHG, 8-OHdG, and HNE-MA (4-hydroxy nonenalmercapturic acid).[117]

All of these molecular interactions indicate that APAP or metabolites of APAP, such as NAPQI, cause oxidative stress, DNA damage, and dose responsive GSH depletion, and these data are consistent with the published OECD AOP No. 20 (**Figure 11. Oxidative Stress and Developmental Impairment of Learning and Memory**).[118] As indicated below, oxidative damage is reported to occur at therapeutic dosages, with dose-effects modified by timing, duration, and frequency of exposures.

2. Cellular Interaction

At the cellular level, APAP and its metabolites cause several key events in the adverse outcome pathway that leads to neurodevelopmental damage, ASD and ADHD.

The APAP metabolite NAPQI causes cellular damage.

Free radicals, such as NAPQI, can bind to molecules within cells and damage or disrupt the normal functions of proteins, lipids, DNA, and other components of the cell, resulting in various types of oxidative-damaging impacts. For example, NAPQI can bind to sulfur-containing amino acids, such as cysteines (Cys), that are the building blocks for proteins. Although Cys residues are commonly targeted,[119] it has also been reported that NAPQI may also damage proteins at methionine (Met), tyrosine (Tyr), and tryptophan (Trp) residues.[120] When NAPQI binds to Cys or other amino acids found in proteins, it can alter a structure or function of the protein.

The primary proteins NAPQI is reported to interact with are glutathione S-transferases.[121] Glutathione S-transferases catalyze the reduction or conjugation of GSH to various electrophilic compounds, including NAPQI. NAPQI is reported to interact with a Cys residue in position 49 of the protein, and alkylation of

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[115] Anand et al. Perinatal Acetaminophen Exposure and Childhood Attention-Deficit/Hyperactivity Disorder (ADHD): Exploring the Role of Umbilical Cord Plasma Metabolites in Oxidative Stress Pathways. Brain Sci. 2021 Sep 30;11(10):1302. doi: 10.3390/brainsci11101302. PMID: 34679367; PMCID: PMC8533963.

[116] Sun et al. Urinary concentrations of acetaminophen in young children in central and south China: Repeated measurements and associations with 8-hydroxy-guanosine and 8-hydroxy-2'-deoxyguanosine. Sci Total Environ. 2021 Sep 15;787:147614. doi: 10.1016/j.scitotenv.2021.147614. Epub 2021 May 8. PMID: 33992949.

[117] Li et al. Urinary paracetamol (4-acetaminophenol) and its isomer 2-acetaminophenol of Chinese pregnant women: Exposure characteristics and association with oxidative stress biomarkers. Sci Total Environ. 2022 Dec 15;852:158375. doi: 10.1016/j.scitotenv.2022.158375. Epub 2022 Aug 29. PMID: 36049689.

[118] Tschudi-Monnet et al. Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leading to impairment of learning and memory, OECD Series on Adverse Outcome Pathways, No. 20, OECD Publishing, Paris, 2022. https://doi.org/10.1787/4df0e9e4-en.

[119] Streeter et al. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro. Chem Biol Interact. 1984 Mar;48(3):349-66. doi: 10.1016/0009-2797(84)90145-5. PMID: 6713598.

[120] Leeminget al. What Are the Potential Sites of Protein Arylation by N-Acetyl-p-benzoquinone Imine (NAPQI)? Chem Res Toxicol. 2015 Nov 16;28(11):2224-33. doi: 10.1021/acs.chemrestox.5b00373. Epub 2015 Nov 2. PMID: 26523953.

[121] Coles et al. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988 Jul;264(1):253-60. doi: 10.1016/0003-9861(88)90592-9. PMID: 3395122.

this residue is accompanied by marked increase in the specific activity of the enzyme.[122] The predominate form of glutathione S-transferase that conjugates NAPQI is referred to as glutathione S-transferase Pi.[123] Absence of glutathione S-transferase Pi is reported to mitigate APAP toxicity, because its absence limits the ability of NAPQI to deplete GSH.[124] NAPQI has also been reported to bind 10-formyltetrahydrofolate dehydrogenase,[125] also known as aldehyde dehydrogenase 1L1 (ALDH1L1), glutamate dehydrogenase,[126] and aldehyde dehydrogenase.[127] The enzyme ALDH1L1, which is involved in folate metabolism, is found in high amounts in the liver.[128] ALDH1L1 participates in one-carbon metabolism by converting10-formyltetrahydrofolate to tetrahydrofolate. Mice lacking the *Aldh1l1* gene exhibit changes in liver metabolism and show symptoms similar to folate deficiency when they were fed a standard mouse diet.[129]

Both glutamate dehydrogenase and aldehyde dehydrogenase are mitochondrial proteins. NAPQI is taken up by mitochondria, which are the organelles within cells that produce ATP (adenosine triphosphate), the molecule that is used to store and transport cellular-chemical energy.[130] The normal operation of mitochondria produces free radicals that must be scavenged, so mitochondria are particularly vulnerable to ROS and oxidative stress.

NAPQI can also disrupt and damage structural proteins. The interior of cells contains various structural or cytoskeletal proteins, including microfilament proteins (e.g., actin), microtubules (e.g., tubulin), and scaffold proteins (e.g., spectrin). These proteins allow organism movements (myosin-actin), cell movements (microtubules), movement of structures within cells, and scaffolding to shape the cell. NAPQI was reported to decrease spectrin and actin proteins.[131] Spectrin is reported to influence cell adhesion and spreading, formation of lamellipodia, and morphogenetic processes, such as eye development, oogenesis,

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[122] Weis et al. N-acetyl-p-benzoquinone imine-induced protein thiol modification in isolated rat hepatocytes. Biochem Pharmacol. 1992 Apr 1;43(7):1493-505. doi: 10.1016/0006-2952(92)90207-y. PMID: 1567474.

[123] Coles et al. The spontaneous and enzymatic reaction of N-acetyl-p-benzoquinonimine with glutathione: a stopped-flow kinetic study. Arch Biochem Biophys. 1988 Jul;264(1):253-60. doi: 10.1016/0003-9861(88)90592-9. PMID: 3395122.

[124] Henderson et al. Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione S-transferase Pi. Proc Natl Acad Sci U S A. 2000 Nov 7;97(23):12741-5. doi: 10.1073/pnas.220176997. PMID: 11058152; PMCID: PMC18834.

[125] Pumford et al. Covalent binding of acetaminophen to N-10-formyltetrahydrofolate dehydrogenase in mice. J Pharmacol Exp Ther. 1997 Jan;280(1):501-5. PMID: 8996234.

[126] Halmes et al. Glutamate dehydrogenase covalently binds to a reactive metabolite of acetaminophen. Chem Res Toxicol. 1996 Mar;9(2):541-6. doi: 10.1021/tx950158a. PMID: 8839060.

[127] Landin et al Identification of a 54-kDa mitochondrial acetaminophen-binding protein as aldehyde dehydrogenase. Toxicol Appl Pharmacol. 1996 Nov;141(1):299-307. doi: 10.1006/taap.1996.0287. PMID: 8917703.

[128] Krupenko. FDH: an aldehyde dehydrogenase fusion enzyme in folate metabolism. Chem Biol Interact. 2009 Mar 16;178(1-3):84-93. doi: 10.1016/j.cbi.2008.09.007. Epub 2008 Sep 19. PMID: 18848533; PMCID: PMC2664990.

[129] Krupenko NI, Sharma J, Pediaditakis P, Fekry B, Helke KL, Du X, Sumner S, Krupenko SA. Cytosolic 10-formyltetrahydrofolate dehydrogenase regulates glycine metabolism in mouse liver. Sci Rep. 2019 Oct 17;9(1):14937. doi: 10.1038/s41598-019-51397-1. PMID: 31624291; PMCID: PMC6797707; Sharma et al. Sex-Specific Metabolic Effects of Dietary Folate Withdrawal in Wild-Type and Aldh1l1 Knockout Mice. Metabolites. 2022 May 18;12(5):454. doi: 10.3390/metabo12050454. PMID: 35629957; PMCID: PMC9143804.

[130] Ramachandran and Jaeschke. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem Toxicol. 2020 Jun;140:111282. doi: 10.1016/j.fct.2020.111282. Epub 2020 Mar 21. PMID: 32209353; PMCID: PMC7254872.

[131] Nicotera et al. Differential effects of arylating and oxidizing analogs of N-acetyl-p-benzoquinoneimine on red blood cell membrane proteins. Arch Biochem Biophys. 1990 Nov 15;283(1):200-5. doi: 10.1016/0003-9861(90)90631-8. PMID: 2146923.

and angiogenesis.[132] Actin and bundles of actin referred to as actin cables are essential to neurulation and neural tube closure.[133]

The oxidative stress-induced damage can disrupt normal neural development in multiple ways. As indicated below, it can disrupt migration of cells or the differentiation of stem cells into neurons, resulting in the production of different types of cells, such as glial cells or astrocytes. It can also impede the proliferation of stem cells directly or cause them to differentiate prematurely, resulting in fewer cells and thereby fewer neurons produced. Overt toxicity can also result in apoptosis or cell death.

A number of studies report the adverse effects of APAP exposure at the cellular level.

In a study using human neural stem cells as a model for developmental neurotoxicity, APAP was included as one of several chemicals for testing. Background information provided by the study resulted in APAP being initially classified as "non-neurotoxic" with therapeutic concentrations of 33-132μM, and toxic concentrations of 165-992 μM.[134] This study confirmed cytotoxicity at the highest concentration of APAP tested, 1000μM == 1mM.

Studies have shown that the APAP metabolite NAPQI is taken up by mitochondria and depletes ATP (adenosine triphosphate). One of the more dramatic impacts of NAPQI (400μM) on cellular energy production was demonstrated by both the rapid depletion of ATP (>80% depletion with a 1-minute exposure) and almost complete inhibition of oxygen consumption in exposed hepatocytes (~90% inhibition with a 10 second exposure).[135] This concentration is 8-10X concentrations found *in vivo*, but NAPQI also has significant biological impacts at lower concentrations.

For example, NAPQI can bind to Topoisomerase II (Topo II) and interfere with its cellular-nuclear functions.[136] Topo II normally changes the topology of DNA (winding, knotting, tangling strands) by cutting and resealing both strands of DNA, allowing DNA strands to pass through each other. This helps to unknot, untangle, link, or unlink DNA molecules during DNA replication, transcription, and chromosome segregation.[137] It has also been proposed that chromatin packing is triggered by histone-dependent, Topo II-mediated clamping of DNA strands.[138] NAPQI stimulates DNA cutting by Topo II,

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[132] Machnicka et al. The role of spectrin in cell adhesion and cell-cell contact. Exp Biol Med (Maywood). 2019 Nov;244(15):1303-1312. doi: 10.1177/1535370219859003. Epub 2019 Jun 21. Erratum in: Exp Biol Med (Maywood). 2019 Jul 17;:1535370219867051. PMID: 31226892; PMCID: PMC6880153.

[133] Galea et al. Biomechanical coupling facilitates spinal neural tube closure in mouse embryos. Proc Natl Acad Sci U S A. 2017 Jun 27;114(26):E5177-E5186. doi: 10.1073/pnas.1700934114. Epub 2017 Jun 12. PMID: 28607062; PMCID: PMC5495245.

[134] Buzanska et al. A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. Stem Cells. 2009 Oct;27(10):2591-601. doi: 10.1002/stem.179. PMID: 19609937.

[135] Andersson et al. N-acetyl-p-benzoquinone imine-induced changes in the energy metabolism in hepatocytes. Chem Biol Interact. 1990;75(2):201-11. doi: 10.1016/0009-2797(90)90118-7. PMID: 2369786.

[136] Bender et al. N-acetyl-p-benzoquinone imine, the toxic metabolite of acetaminophen, is a topoisomerase II poison. Biochemistry. 2004 Mar 30;43(12):3731-9. doi: 10.1021/bi036107r. PMID: 15035644.

[137] Reviewed in Deweese et al. DNA Topology and Topoisomerases: Teaching a "Knotty" Subject. Biochem Mol Biol Educ. 2008;37(1):2-10. doi: 10.1002/bmb.20244. PMID: 19225573; PMCID: PMC2643378.

[138] Hizume et al. Topoisomerase II, scaffold component, promotes chromatin compaction in vitro in a linker-histone H1-dependent manner. Nucleic Acids Res. 2007;35(8):2787-99. doi: 10.1093/nar/gkm116. Epub 2007 Apr 11. PMID: 17430970; PMCID: PMC1885653.

>5-fold at 100 μM, but repair of the cuts was inhibited by NAPQI.[139] APAP has also shown inhibitory effects on DNA repair in multiple mammalian cells.[140] Cytotoxicity and DNA damage were also examined in hepatoma cells, and results indicated NAPQI produces DNA and cellular damage (Table 4). The cytotoxicity observed at 24hrs with the lowest concentration of NAPQI tested, 0.05mM / 50μM, poses a relevant risk. As indicated below, 1mM APAP is a relevant *in vivo* concentration found in plasma and brains of animals exposed to APAP at therapeutic human equivalent doses.[141] With a concentration of 1mM APAP, then 5-15% would be expected to be oxidized into NAPQI, resulting in NAPQI concentrations of 50-150mM, which produced cytotoxicity.

Test substance		Elution rate constant	Cytotoxicity (% of control)	
			1 h	24 h
DMSO (control)		0.010 ± 0.002	0 ± 0	0 ± 0
Paracetamol	10 mM	0.012 ± 0.004	0 ± 0	0 ± 0
NAPQI	0.05 mM	0.041 ± 0.019	0 ± 0	36 ± 11
	0.10 mM	0.075 ± 0.036	2 ± 2	81 ± 3
	0.25 mM	0.173 ± 0.068	5 ± 1	100 ± 0
N-OH-AAF	0.10 mM	0.155 ± 0.029	0 ± 0	0 ± 0

Table 4. NAPQI Cytotoxicity. Reuber hepatoma cells, prelabelled with 3H-TdR, were exposed to test substances for 1 h. The cultures were then rinsed and prepared for the alkaline elution assay or cytotoxicity was determined immediately or after incubation for an additional 23 h. Means +/- S.D. of 4-8 determinations. APAP does not show cytotoxicity relative to DMSO control. The APAP metabolite NAPQI is cytotoxic and resulted in DNA damage at all tested concentrations (0.05mM-0.25mM) at 24h. [142]

The bulk of APAP toxicity is focused on hepatotoxicity, where APAP has also been shown to produce mitochondrial oxidative and nitrosative stress.[143] The mitochondrial alterations caused by APAP are well-established in the scientific literature and have been recently reviewed.[144] Briefly, the mitochondria are organelles in the cell that produce energy in the form of ATP. The process of producing ATP involves the transfer of electrons from one protein complex to another in the electron transport chain (ETC). During this process, free radicals such as superoxide are generated, but they are normally-efficiently scavenged by superoxide dismutases (SOD) within the mitochondria. However, when mitochondria are exposed to NAPQI, NAPQI can bind with mitochondrial proteins and form "adducts," which are combinations of NAPQI and the protein. Adduct formation on mitochondrial proteins such as ATP synthase can result in reverse electron transport within the electron transport chain, which elevates superoxide generation and induces oxidative damage. Protein adduct formation on antioxidant enzymes prevents efficient scavenging of superoxide, which enables nitric oxide to form peroxynitrite radicals. These radicals can also modify

[139] Bender et al. N-acetyl-p-benzoquinone imine, the toxic metabolite of acetaminophen, is a topoisomerase II poison. Biochemistry. 2004 Mar 30;43(12):3731-9. doi: 10.1021/bi036107r. PMID: 15035644.

[140] Brunborg et al. Inhibitory effects of paracetamol on DNA repair in mammalian cells. Mutat Res. 1995 Apr;342(3-4):157-70. doi: 10.1016/0165-1218(95)90025-x. PMID: 7715617.

[141] Posadas et al. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. doi: 10.1371/journal.pone.0015360. PMID: 21170329; PMCID: PMC3000821.

[142] Dybing et al. Genotoxicity studies with paracetamol. Mutat Res. 1984 Oct;138(1):21-32. doi: 10.1016/0165-1218(84)90081-8. PMID: 6387477.

[143] Ramachandran and Jaeschke. Acetaminophen hepatotoxicity: A mitochondrial perspective. Adv Pharmacol. 2019;85:195-219. doi: 10.1016/bs.apha.2019.01.007. Epub 2019 Feb 21. PMID: 31307587; PMCID: PMC7350903.

[144] Ramachandran and Jaeschke. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem Toxicol. 2020 Jun;140:111282. doi: 10.1016/j.fct.2020.111282. Epub 2020 Mar 21. PMID: 32209353; PMCID: PMC7254872.

enzymes such as SOD as well as spill out of the mitochondria into the cytosol resulting in mitochondrial and cytoplasmic interactions and toxicity.

Genetic toxicity was also reported in the NTP carcinogenesis and genetic toxicity studies of APAP. This included negative results in Ames testing using *Salmonella typhimurium*, and positive genetic toxicity findings for sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells.[145] This is in contrast to the leukemias reported by NTP. It was also reported independently by a research group Boehringer Ingelheim Pharmaceuticals that the APAP metabolite (p-Aminophenol, PAP) resulted in reproducible dose-related increases in cells with single-strand DNA breaks with both the mouse lymphoma cells (L5178Y) and the Chinese hamster ovary (CHO) cells.[146]

The damage from NAPQI has been reported to cause apoptosis (cell death) in rat cortical neurons.[147] APAP intoxication has also been reported to produce astrogliosis, an abnormal increase in astrocytes due to destruction of local neurons, in areas of the brain associated with locomotion regulation and to havea direct toxic effect on a mixed primary culture of two types of brain cells, astrocytes and oligodendrocytes.[148] This study also reported that APAP has a direct neurotoxic effect on the selective modulation of dopamine content in motor-regulating regions of the brain due to downregulation of tyrosine hydroxylase expression. This is the first study to provide evidence of a mechanistic link between toxic APAP dosing and direct adverse effects on glial components of the CNS.

In addition, the inhibitory neurons known as Purkinje cells are necessary for well-coordinated movement, cognition, and emotion. They regulate the activation of excitatory neurons by interactions and release gama-aminobutyric acid (GABA), a neurotransmitter that inhibits certain neurons from transmitting impulses. These cells are vulnerable to oxidative stress, and some forms of autism have significant loss of Purkinje cells in the cerebellum.[149]

APAP Disrupts the Endocannabinoid Signaling Essential for Brain Development.

The endocannabinoid cell-signaling system (ECS) includes cannabinoid receptors CB1 (CB1R) and CB2 (CB2R), the endocannabinoid agonists (activators) anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes that synthesize and metabolize the endocannabinoid ligands.[150]

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[145] National Toxicology Program. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 1993 Jan;394:1-274. PMID: 12637965.

[146] Majeska and Holden. Genotoxic effects of p-aminophenol in Chinese hamster ovary and mouse lymphoma cells: results of a multiple endpoint test. Environ Mol Mutagen. 1995;26(2):163-70. doi: 10.1002/em.2850260210. PMID: 7556113.

[147] Posadas I, Santos P, Blanco A, Muñoz-Fernández M, Ceña V. Acetaminophen induces apoptosis in rat cortical neurons. PLoS One. 2010 Dec 10;5(12):e15360. Doi:10.1371/ journal.pone.0015360.

[148] Vigo et al. Acute acetaminophen intoxication induces direct neurotoxicity in rats manifested as astrogliosis and decreased dopaminergic markers in brain areas associated with locomotor regulation. Biochem Pharmacol. 2019 Dec;170:113662. doi: 10.1016/j.bcp.2019.113662. Epub 2019 Oct 10. PMID: 31606411.

[149] Tamiji and Crawford. The neurobiology of lipid metabolism in autism spectrum disorders. Neurosignals. 2010;18(2):98-112. doi: 10.1159/000323189. Epub 2011 Feb 4. PMID: 21346377.

[150] Maccarroneet al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015 May;36(5):277-96. doi: 10.1016/j.tips.2015.02.008. Epub 2015 Mar 18. PMID: 25796370; PMCID: PMC4420685.

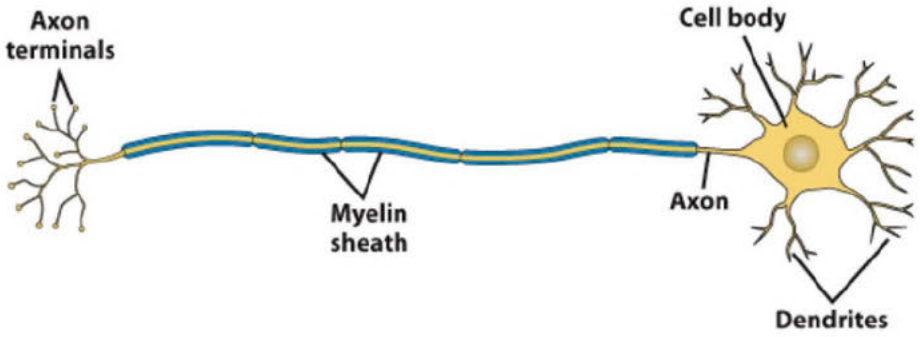

Figure 14. Schematic diagram of an idealized neuron with major components.

Neurons come in a variety of types, such as serotonergic or dopaminergic, but are collectively specialized cells that transmit information throughout the nervous system. They have a cell body and branching structures called dendrites and axons (Figure 14). Dendrites receive signals from other neurons, while axons send signals to other neurons. The point where an axon terminal of one neuron meets the dendrite of another neuron is called a synapse. Neuronal signaling occurs when an electrical signal travels from the cell body along the axon to the axon terminal, where it triggers the release of neurotransmitters. These neurotransmitters cross the synaptic cleft and bind to receptors on the dendrite of the next neuron, transmitting the signal. There are more than 100 known neuropeptide neurotransmitters in the human nervous system.[151] These include glutamate, GABA (gamma-aminobutyric acid), acetylcholine, glycine, and norepinephrine, dopamine, serotonin, histamine, and endocannabinoids. Endocannabinoids are reported to regulate a variety of physiological processes, including cardiovascular, gastrointestinal, and hepatic (liver) functions, adaptive and innate immunity, muscle formation, bone remodeling, pain sensation, mood, and memory.[150]

The endocannabinoid cell-signaling system (ECS) targets two receptors known as the CB1 and CB2 cannabinoid receptors. The neurotransmitter molecule that is the activator (or agonist) for the CB1 receptor is anandamide (an endogenous cannabinoid). The activator/agonist for the CB2 receptor is 2-arachidonoylglycerol (2-AG).[152] Studies have also suggested the occurrence of other endocannabinoid targets beyond CB1 and CB2, proposing GPR55 and GPR35 share common interactions through a metabolite of 2-AG, 2-arachidonoyl lysophosphatidic acid (LPA).[153]

APAP is reported to cause CB1 and CB2 activation through several mechanisms. APAP can be metabolized via hydrolysis to form p-aminophenol (PAP). Then PAP undergoes conjugation with arachidonic acid, using the enzyme fatty acid amid hydrolase (FAAH), to produce N-acylphenolamine

[151] Cuevas. Neurotransmitters and their Life Cycle. xPharm: The Comprehensive Pharmacology Reference. 1-7. 2011. doi: 10.1016/B978-008055232-3.60016-9.
[152] Maccarroneet al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015 May;36(5):277-96. doi: 10.1016/j.tips.2015.02.008. Epub 2015 Mar 18. PMID: 25796370; PMCID: PMC4420685.
[153] Zhao and Abood. GPR55 and GPR35 and their relationship to cannabinoid and lysophospholipid receptors. Life Sci. 2013 Mar 19;92(8-9):453-7. doi: 10.1016/j.lfs.2012.06.039. Epub 2012 Jul 20. PMID: 22820167.

(AM404).[154] The AM404 metabolite increases brain endocannabinoid levels by decreasing the re-uptake of anandamide. The result is an increased level of anandamide.[155] In addition, the chemical structure of AM404 is similar to anandamide (Figure 15).[156] Because of these similarities, AM404 can bind with and activate the CB1 receptor (although it is a weaker agonist than anandamide).[155] AM404 is also reported be an agonist (activator) of the CB2 receptor.[157] CB2 is expressed in peripheral and immune tissues, and animal studies have detected it in various brain regions, including olfactory tubercle, islands of Calleja, cerebral cortex, striatum, thalamic nuclei, hippocampus, amygdala, substantia nigra, periaqueductal gray, paratrochlear nucleus, paralemniscal nucleus, red nucleus, pontine nuclei, inferior colliculus and the parvocellular portion of the medial vestibular nucleus.[158]

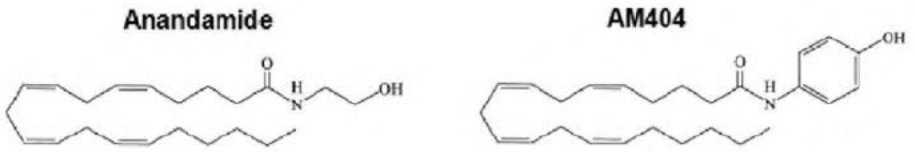

Figure 15. Structures of Endocannabinoid Anandamide and Acetaminophen Metabolite AM404. Both molecules share a common 20-carbon backbone derived from arachidonic acid.

At typical therapeutic doses, APAP and/or the metabolite AM404 mediate endocannabinoid signaling. This effect is considered to be a primary mechanism of action by which APAP relieves pain.[155] For example, experiments have demonstrated that disrupting the CB1 receptor in mice or rats (either genetically or through pharmacological antagonist) results in loss of the analgesic activity of APAP.[159,155] In addition, inhibition of FAAH will suppress the analgesic effect of acetaminophen, which indicates that acetaminophen's pain modification depends on production of AM404 which makes use of FAAH.[159] In one notable case study, a Scottish woman who felt no pain was reported to lack the gene that produces FAAH, clearly demonstrating the analgesic impact of this molecular pathway.[160]

While these pharmacological effects on the endocannabinoid system are useful in adults as a temporary analgesic or antipyretic, this same pathway is essential for brain development, and disruption of it during

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[154] Högestätt et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005 Sep 9;280(36):31405-12. doi: 10.1074/jbc.M501489200. Epub 2005 Jun 29. PMID: 15987694.

[155] Bertolini, A., Ferrari, A., Ottani, A., Guerzoni, S. Tacchi, R., Leone, S., 2006, paracetamol: new vistas of an old drug. CNS Drug Rev. 12 (3-4), 250-275, p. 255.

[156] Ghanen, C., Perez Mariz, J., Manautou, J., Mottino, A., Acetaminiophen; From liver to brain: new insights into drug pharmacological action and toxicity, Pharmacol Res. 2016 July; 109: 119-131, DOI: 10.1016/ j.phrs.2016.02.020, p. 3.

[157] Buhrer C, Endesfelder S, Scheuer T, Schmitz T, Paracetamol (Acetaminophen) and the Developing Brain, Int. J. Mol. Sci. 2021, 22, 11156, DOI: 10.3390/ ijms222011156, p.4.

[158] Gong et al. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res. 2006 Feb 3;1071(1):10-23. doi: 10.1016/j.brainres.2005.11.035. Epub 2006 Feb 9. PMID: 16472786.

[159] Mallet et al. Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. Pain. 2008 Sep 30;139(1):190-200. doi: 10.1016/j.pain.2008.03.030. Epub 2008 May 15. PMID: 18485596.

[160] Habib et al. Microdeletion in a FAAH pseudogene identified in a patient with high anandamide concentrations and pain insensitivity. Br J Anaesth. 2019 Aug;123(2):e249-e253. doi: 10.1016/j.bja.2019.02.019. Epub 2019 Mar 28. PMID: 30929760; PMCID: PMC6676009.

critical windows of brain development can thereby disrupt neurodevelopment. This is because the endocannabinoid system is required for normal brain development.[161]

Endocannabinoids regulate synaptogenesis, axon growth and positioning, and neuronal cell fate.[162] They also regulate both the number and division rate of stem cells that differentiate into neural cells and the type of neuronal cell (neuron or glia) produced from neural stem cells.[161] Endocannabinoids also guide the migration of the neural cells "to their final positions in the cerebral cortex and other brain areas..." and after the cells have arrived at their final location, endocannabinoid molecules affect how the cells build neuronal networks. [162]

It has also been reported that there is a transient window when the dominant brain cannabinoid receptor, CB1, is expressed on afferent terminals (the nerve fibers that extend from the brain to sensory receptors such as on the skin) instead of on the Purkinje neuron cell synapses that dominate the adult cerebellum. The activation of these afferent CB1 receptors during development suppresses synaptic transmission onto developing granule cells (the most common type of neuron in the brain) and consequently suppresses excitation of downstream neurons in the developing cortical network, including nonsynaptic, migrating neurons.[163] This study also reports:

> …there is a transient period in the newborn rodent, during the cerebellar "growth spurt," when functional CB1Rs are expressed on MF (mossy fiber) afferent terminals and on inhibitory interneuron cross-connected synapses, but not on the excitatory or inhibitory afferents to PCs (Purkinje cells) that dominate in the adult... (inserted)

A direct analogy can be made between fetal exposure to APAP and fetal exposure to marijuana. This is supported by evidence that phyto-based (plant) cannabinoids, such as delta-9-tetrahydrocannabinol (Δ9THC), are reported to have an impact on neurodevelopment similar to that seen in exposure to APAP. Exposing mice to THC on post-natal day 10 (corresponding to end of third-trimester exposure in humans, at peak brain growth spurt) results in altered spontaneous behavior and habituation rates in adult mice.[164] Similar behavioral alterations occur when mice are exposed to APAP on PND10.[165, 166] Because THC and APAP both interact with the endocannabinoid system, it has been hypothesized that this system might be involved in the same adverse outcome pathway for both of these drugs.[167]

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[161] Harkany and Cinquina. Physiological Rules of Endocannabinoid Action During Fetal and Neonatal Brain Development. Cannabis Cannabinoid Res. 2021 Oct;6(5):381-388. doi: 10.1089/can.2021.0096. Epub 2021 Oct 6. PMID: 34619043; PMCID: PMC8664114.

[162] Gaffuri et al. Type-1 cannabinoid receptor signaling in neuronal development. Pharmacology. 2012;90(1-2):19-39. doi: 10.1159/000339075. Epub 2012 Jul 3. PMID: 22776780.

[163] Barnes et al. Developmentally Transient CB1Rs on Cerebellar Afferents Suppress Afferent Input, Downstream Synaptic Excitation, and Signaling to Migrating Neurons. J Neurosci. 2020 Aug 5;40(32):6133-6145. doi: 10.1523/JNEUROSCI.1931-19.2020. Epub 2020 Jul 6. PMID: 32631938; PMCID: PMC7406284.

[164] Philippott, G., Forsberg, E., Tahan, C., Viberg, H., & Fredriksson, R. A single Δ9-Tetrahyddrocannabinol THC) dose during brain development affects markers of neurotrophy, oxidative stress, and apoptosis (2019) Front. Pharmacol. 10:1156, DOI:10.3389/ fphar.2019.01156

[165] Viberg et al. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. Toxicol Sci. 2014 Mar;138(1):139-47. doi: 10.1093/toxsci/kft329. Epub 2013 Dec 21. PMID: 24361869.

[166] Philippot et al. Adult neurobehavioral alterations in male and female mice following developmental exposure to paracetamol (acetaminophen): characterization of a critical period. J Appl Toxicol. 2017 Oct;37(10):1174-1181. doi: 10.1002/jat.3473. Epub 2017 Apr 27. PMID: 28448685.

[167] Philippott, G., Forsberg, E., Tahan, C., Viberg, H., & Fredriksson, R. A single Δ9-Tetrahyddrocannabinol THC) dose during brain development affects markers of neurotrophy, oxidative stress, and apoptosis (2019) Front. Pharmacol. 10:1156, DOI:10.3389/ fphar.2019.01156